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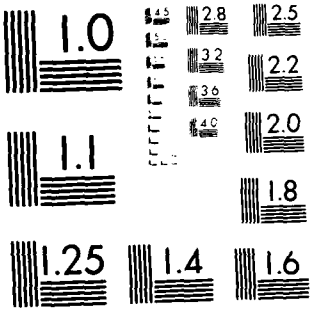

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Annual Report

From 3/1/80 - 11/26/80 ONR Contract N00014-79-C-0242

Enzymatic Conversion of Red Cells for Transfusion

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Enzymatic Studies

Alpha Galactosidase

As part of our ongoing studies of the properties of the exo-alpha galactosidase from green coffee beans, we have completed comparative kinetic measurements of the removal of cell surface B antigenicity by this enzyme both in the free state and when covalently attached to soluble dextran. Typical results are presented in Table I and include treatment of B erythrocytes with either alpha galactosidase, alpha galactosidase dextran conjugate or just free enzyme in the presence of dextran. Regardless of the kind of enzyme treatment, increased H antigenic titers were produced which are equivalent to those found with type O cells. Erythrocytes incubated with alpha galactosidase yielded a zero agglutination score at 70' while those treated with alpha galactosidase dextran were found to be non-agglutinable with Anti B in 45', demonstrating that the enzyme dextran conjugate removes B antigenicity at a faster rate than free enzyme. Interestingly, the addition of dextran to free enzyme increased the rate to that of the enzyme conjugate suggesting that dextran itself facilitates the interaction of the enzyme with the surface of the red cell. We have also prepared and stockpiled enzyme dextran conjugate and purified free enzyme in amounts sufficient to inaugurate the clinical studies described in the Renewal Proposal.

Alpha N-acetylgalacotosaminidase

Last year we reported the isolation of an alpha N-acetylgalactosaminidase (A-zyme) free of neuraminidase activity from Aspergillus niger. Although reported to be active with blood group substances, it was unable to remove A antigenicity from red cells under our conversion conditions. We are currently working with an enzyme present in the limpet Patella vulgata. This enzyme has been reported to destroy A blood group activity present in blood group substances when used at pH 4. However, it becomes progressively inactive with increasing pH which is thought to be due to the enzyme's dissociating into subunits. We have isolated the enzyme and have shown that it can remove A acitvity from preparations of red cell membranes at pH 4. We have prepared a derivative of dextran which will now allow us to attempt to bind the intact enzyme to dextran at pH 4. Under these conditions, the molecule should be unable to dissociate into subunits and thus will presumably retain activity at the higher pH needed to remove antigenic activity from red cells while maintaining their viability.

Metabolic, Structural and In Vivo Survival Properties of Enzymatically Converted Type B Erythrocytes

Gibbon Studies

We are close to completing our studies with gibbon red cells which have a B-like antigen and thus provide an experimental model that can be used to preview the fate, in vivo, of enzyme treated cells before attempting similar human studies. Under our current enzyme treatment conditions gibbon cells lose all agglutinability with human anti B antiserum within 1-2 hours. When fragility studies are performed with such cells, the results shown in Table II and Figure 1 are obtained which demonstrate that

TABLE I

RATE OF LOSS OF B ANTIGENIC ACTIVITY FROM ENZYME-TREATED CELLS.

	HEMAGGLUTINATION SCORE AT:				
TYPE B ERYTHROCYTES INCUBATED WITH	15′	30′	45′	60′	70′
∝-GALACTOSIDASE	12	11	9	4	0
∝-GALACTOSIDASE-DEXTRAN	12	3	0	0	0
∝-GALACTOSIDASE PLUS DEXTRAN	12	2	0	0	0
BUFFER	12	12	12	12	12

ALL ENZYME TREATMENTS RESULTED IN INCREASED H ANTIGENIC TITERS EQUIVALENT TO THOSE FOUND WITH TYPE O CELLS.

enzyme treatment neither affects the cells susceptibility to osmotic shock nor renders any part of the cell population more fragile since in the latter case the shapes of the curves for untreated and treated cells are quite similar without any hemolysis occurring at sodium chloride concentrations between 0.9% - 0.6%. We next looked for changes in the metabolic capabilities of converted cells, particlarly with respect to maintenance of normal levels of adenosine 5'-triphosphate (ATP) which appears to govern cell deformability and 2,3 diphosphoglyceric acid (2,3 DPG) which provides for normal oxygen binding and exchange. The results are shown in Table III and indicate that treatment for 1 and 2 hours produced cells that retained 90% or greater of their ATP content and over 80% of their 2,3 DPG with no difference observed between buffer treated cells and those treated with enzyme. We also have not found any significant increases in methemoglobin levels of treated cells. Furthermore, the levels of two markers for possible membrane alterations, the enzyme acetylcholinesterase which is believed to be bound to the outer membrane surface and cholesterol which is involved, among other things, in the maintenance of proper membrane fluidity, remained unchanged following enzyme treatement.

These results encouraged us to perform in vivo survival studies. The results of three such recent experiments are given in Table IV. Cells were labeled with Cr^{51} as described in last year's report and returned to the donor animal as in the case of the pH 7.4 buffer control, or subsequently treated with either alpha galactosidase or alpha galacotsidase dextran conjugate, and then returned to the donor animal. Following intravenous administration of the labeled cells, samples were collected at 30' and 60', radioactivity was measured and used as the zero time 100% survival value. We have recently taken earlier sampling times (3' and 10') as well as 30' and 60' times, compared them with each other as well as with the amount of radioactivity administered and confirmed that 30' and 60' times were a true reflection of 100% survival. Further samples were taken at appropriate time intervals ranging from 1 to 21 days and yielded essentially linear survival rates as shown in Figure 2. As seen in Table IV, over 90% of the control, alpha galactosidase and alpha galactosidase dextran treated cells remain in the circulation 24 hrs. after being returned to the donor. The biological half life of the treated cells ranges from 14.5 to 17.5 days, times which fall within the normal gibbon erythrocyte biological half life of 14 to 18 days. Thus, these results demonstrate that when enzymatically treated cells are returned to the donor they exhibit normal in vivo survival rates. Furthermore, as part of a continuing surveillance, we are so far unable to detect the formation of antibody toward enzyme treated cells, even after returning such cells to the same donor animal three times, indicating that under these conditions they may not be immunogenic. Also, no antibodies have as yet been detected in these animals to either free enzyme or enzyme dextran conjugates.

Human Studies

Comparable studies to those performed in vitro with treated gibbbon red cells have been done with human cells. Last year we reported that treated cells showed no change in susceptibility to osmotic shock nor, as with gibbon cells, was any part of the cell population rendered more fragile. The results of ATP and 2,3 DPG studies are shown in Table V. Under our conditions, that is, pH 5.7 to 5.8 at 26°, ATP levels remained essenti-

Table II

% NaCl Producing
50% Hemolysis of Gibbon Red Cells

Untreated cells	0.42
Buffer	0.43
α-Galactosidase	0.43

FIGURE 1. FRAGILITY OF TREATED GIBBON ERYTHROCYTES

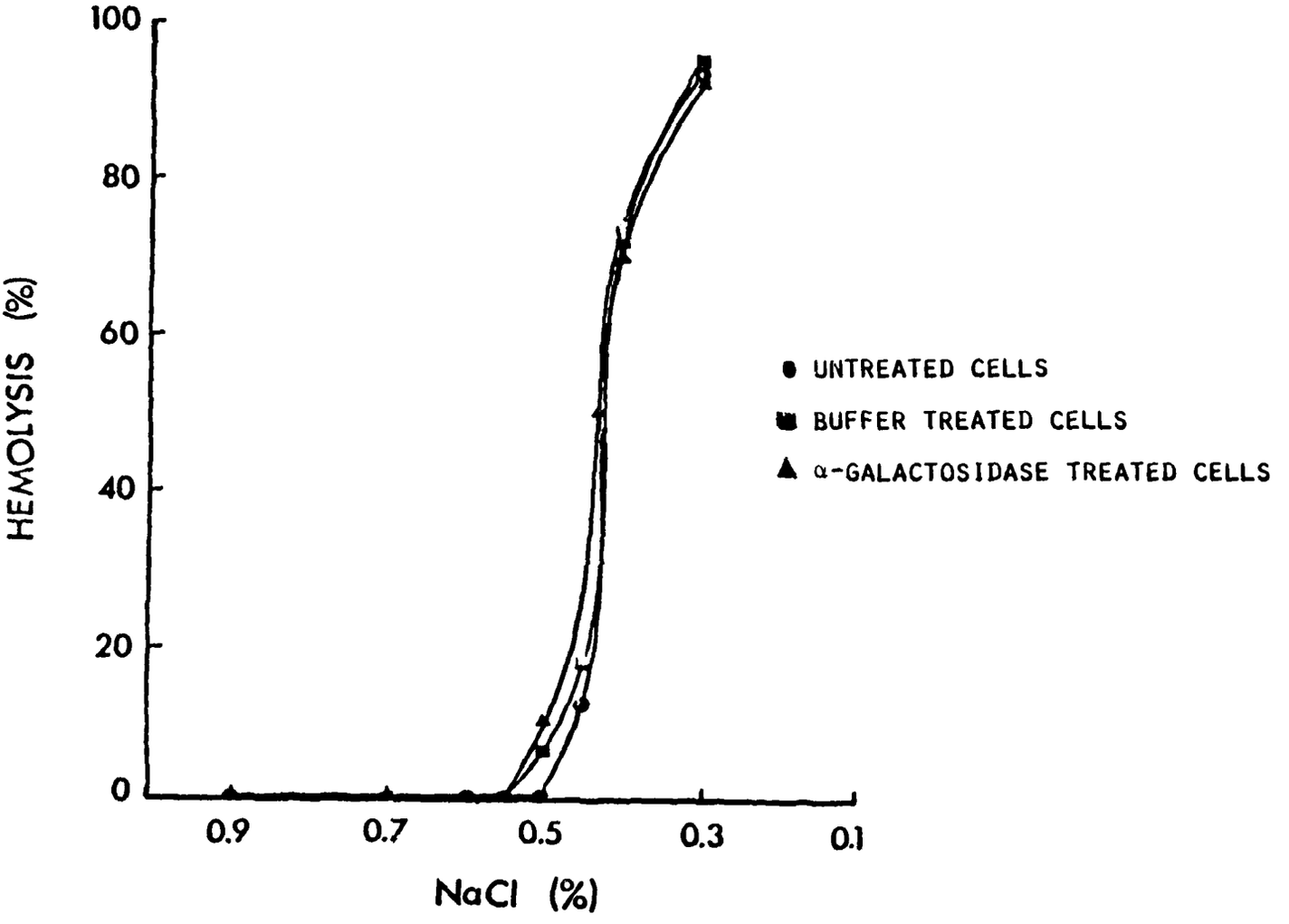


TABLE III

GIBBON ERYTHROCYTE ADENOSINE-5'-TRIPHOSPHATE AND 2,3-DIPHOSPHOGLYCERATE LEVELS FOLLOWING TREATMENT

TREATMENT CONDITIONS		% OF UNTREATED CELLS ATP	2,3-DPG
∝-GALACTOSIDASE	1 HR	92	84
	2 HR	100	88
BUFFER	1 HR	90	83
	2 HR	94	81

TABLE IV

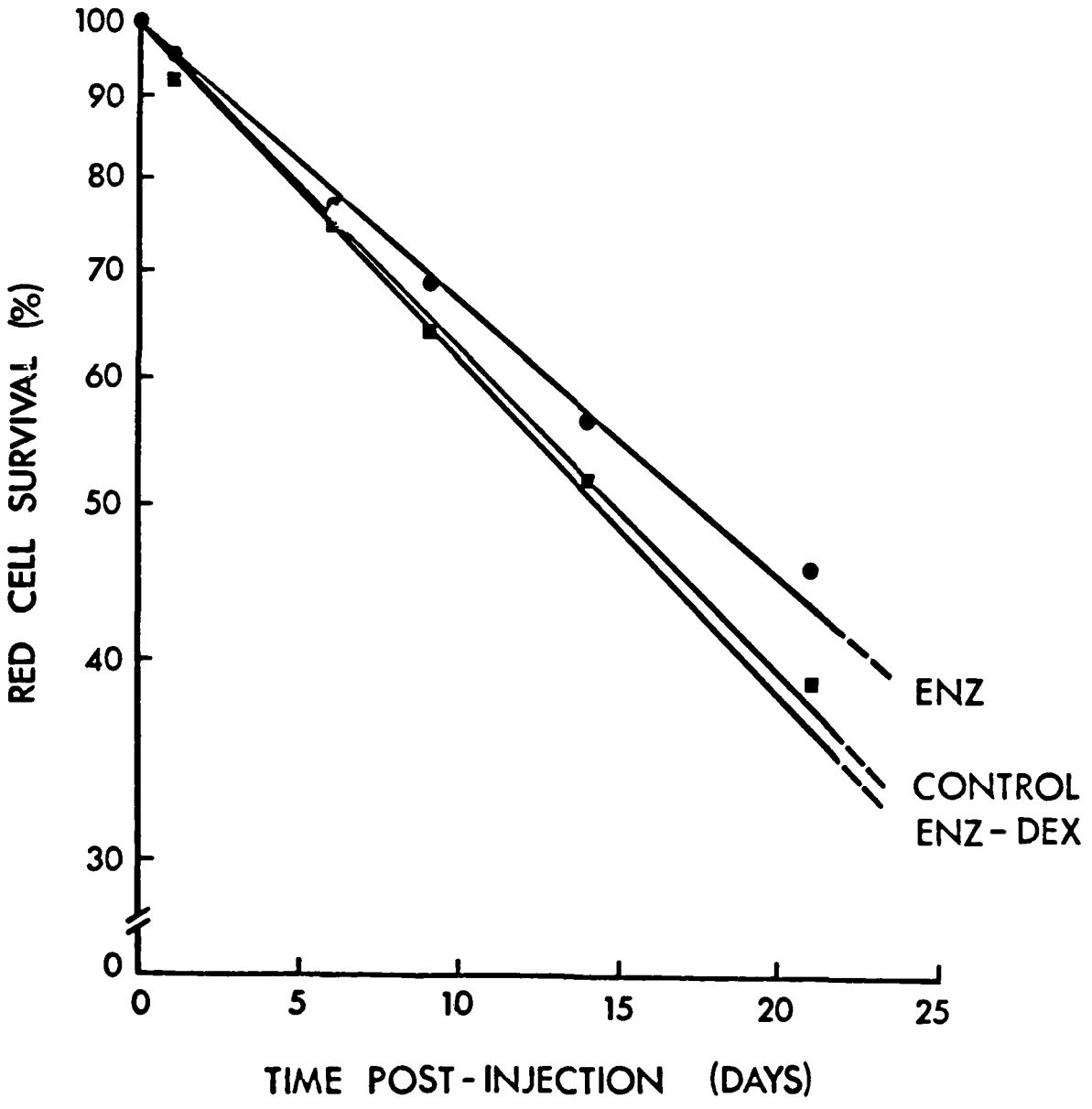
^{51}Cr IN VIVO SURVIVAL OF TREATED GIBBON ERYTHROCYTES*

TREATMENT	24 HR. SURVIVAL (%)	T 1/2 (DAYS)
CONTROL	91	15
∝-GALACTOSIDASE	95	17.5
∝-GALACTOSIDASE-DEXTRAN CONJUGATE	91	14.5

*GIBBON NORMAL ERYTHROCYTE T 1/2 = 14-18 DAYS

FIGURE 2

^{51}Cr *In vivo* SURVIVAL CURVES OF TREATED GIBBON ERYTHROCYTES



ally unchanged for as long as 5 hours of treatment with phosphate-citrate-saline buffer whereas 2,3 DPG levels decreased to 39% of untreated cells at 6 hrs. However, the 2,3 DPG levels remain relatively high for the first 3 hours of such treatment. Most importantly, the results of 1 h incubation with alpha galactosidase at concentrations that remove all antigenic reactivity within that time or similar incubations with 15% and 30% dextran having a molecular weight of 40,000 as well as alpha galactosidase dextran conjugate at the same dextran concentrations, all show ATP and 2,3 DPG levels which remain essentially normal. These results suggest that under free or bound enzyme treatment conditions which remove all antigenic reactivity in 1-2 hrs. cell deformability and oxygen binding and exchange remain unimpaired. This is further supported by our finding a lack of increase in methemoglobin formation under these conditions. Treated human erythrocytes, as did their gibbon counterparts, appear to have not sustained any major membrane damage since they too retain normal levels of acetylcholinesterase and cholesterol, as well as sialic acid. In collaboration with Laurie Marsh, our institution's resident immunohematologist, we have subjected converted cells to extensive antigen profiling as another parameter for membrane change; this is indicated in Figure 3. Included in this group of antigens tested for are: A, B, Rh, MNS, P_1, Lewis, Kell, Lutheran, Duffy and Kidd. Several batches of untreated control and treated cells containing different permutations of these antigens were examined for changes in their expression. Those that were originally present on untreated cells remained on their enzyme treated counterparts, and did not show any detectable weakening. Similarly, those antigens which were intially absent did not appear after enzyme treatment. Only B, and if present, P_1 antigenicity - whose activity is also due to a terminally linked alpha galactose residue - were lost after conversion. These results, although they by no means completely encompass the whole spectrum of testable antigens, would suggest that cryptic antigens have not been uncovered or new antigens formed from changes in pre-existing ones as a result of enzymatic treatment. The only contra-indication to this we have found, is that when unusually stringent detection conditions are employed in the hemagglutination assay such as 1' high speed centrifugation and extremely gentle resuspension of the pelleted cells, we sometimes observe very weak clumping of treated cells with some lots of commercial anti A, B antisera and some individual high hemolysin titer containing sera from group O persons who have been hyperimmmunized against A and/or B antigens. Since no hemolysis of enzyme treated cells occurs at 37° with the high hemolysin sera, whereas control group B cells are completely hemolyzed and the weak agglutination reaction remains the same or becomes even weaker at that temperature, it is not readily apparent what effect, if any, this observation would have on the survival of converted cells and their potential immunogenicity and should be resolved by <u>in vivo</u> studies.

Therefore, taking into account our <u>in vitro</u> results with human cells together with both the <u>in vitro</u> and <u>in vivo</u> results obtained with gibbon cells, we feel we have provided the basis for performing similar <u>in vivo</u> survival studies with converted human erythrocytes - but proceeding one step further. That is, we plan to return treated cells to the group B donor and administer them to group O and A individuals as well.

TABLE V

HUMAN ERYTHROCYTE ADENOSINE 5' TRIPHOSPHATE AND 2,3-DIPHOSPHOGLYCERIC ACID LEVELS FOLLOWING TREATMENT

TREATMENT CONDITIONS		% OF UNTREATED CELLS ATP	% OF UNTREATED CELLS 2,3-DPG
BUFFER	1 HR	101	86
	2 HR	101	75
	3 HR	101	73
	4 HR	99	60
	5 HR	99	51
	6 HR	109	39
∝-GALACTOSIDASE	1 HR	92	91
DEXTRAN 15%	1 HR	105	84
DEXTRAN 30%	1 HR	104	90
∝-GALACTOSIDASE-DEXTRAN 15%	1 HR	100	87
∝-GALACTOSIDASE-DEXTRAN 30%	1 HR	99	90

FIGURE 3

ENZYMATICALLY CONVERTED B CELLS HAVE BEEN TESTED FOR THE FOLLOWING 25 ANTIGENS: A, B, C, D, E, c, e, C^W, M, N, S, s, P_1, Le^A, Le^B, K, k, Kp^A, Kp^B, Lu^B, Lu4, Fy^A, Fy^B, Jk^A, AND Jk^B.

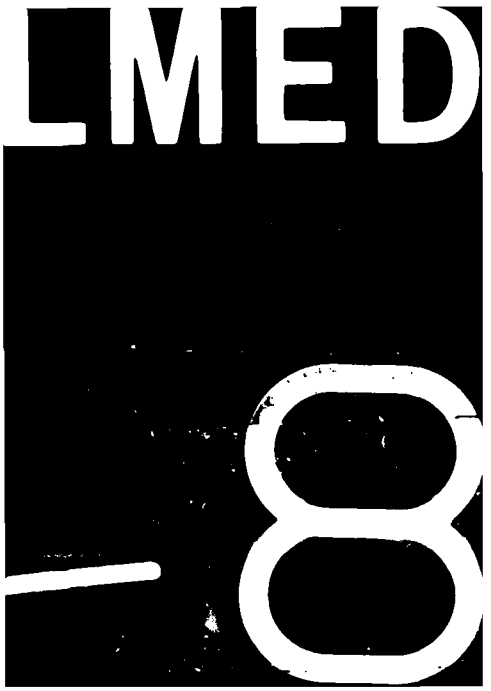
Figure Three is a Viewgraph and is complete per Dr. Majde, ONR/Code 441

Publications List

"Removal of Erythrocyte Surface Antigens by an α-Galactosidase and its Dextran Conjugate," J. Goldstein, J. Y. Kuo, L. Lenny. Joint Meeting of the 18th Cong. International Soc. of Hematology & 16th Cong. International Soc. Blood Transfusion, Montreal, Canada. Abstracts. p. 298, #1634, 1980.

"Nonantigenic Red Blood Cells". J. Goldstein in "Current Concepts of Combat Casualty Resuscitation Symposium". In Press.

"Enzymatic Removal of Blood Group B Antigens from Gibbon Erythrocytes". L.L. Lenny and J. Goldstein. Transfusion, 20, 618, 1980.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